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Structure of the Two Promoters of the Human *lck* Gene: Differential Accumulation of Two Classes of *lck* Transcripts in T Cells

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The human T-cell- or lymphocyte-specific gene, *lck*, encodes a tyrosine kinase and is a member of the *src* family. In this report we demonstrate that there are two classes of human *lck* transcripts (types I and II), containing different 5′-untranslated regions, which are expressed from two distinct promoters. No apparent sequence similarity was observed between the 5′-flanking regions of the two promoters. The expression of *lck* in human T-cell leukemia and carcinoma cell lines and in human peripheral blood T lymphocytes was examined by S1 nuclease and primer extension mapping and by Northern (RNA) blot analysis of total cellular RNA. The following results were obtained. (i) Two RNA start sites in the downstream promoter were used to generate type I transcripts. (ii) The major human type I start site has not been described for the mouse. (iii) At least five RNA start sites in the upstream promoter were used to generate type II transcripts. (iv) In T cells and in two colon carcinoma cell lines, type II transcripts were present in higher amounts than type I transcripts. (v) In T cells treated with phytohemagglutinin, tetradecanoylphorbol acetate, and cyclosporin A, the modulation of *lck* expression was associated primarily with changes in levels of type II transcripts. The above results suggest that the two human *lck* promoters are utilized differentially and may be regulated independently during certain physiological states.

The T-cell- or lymphocyte-specific tyrosine kinase *lck* is a recently described member of the *src* gene family (3, 5, 9, 10, 15, 18, 25). The product of the *lck* gene, $p56^{lck}$, is found mainly associated with T lymphocytes and is expressed at elevated levels in certain T-cell leukemic and lymphoma cell lines (10, 11, 18, 22, 25). This increased level of *lck* has led to its isolation in both murine and human T-lymphoma cells (10, 18). The human *lck* gene is also expressed in colon carcinoma and other nonlymphoid tumor cell lines (22). The expression of *lck* message or protein (or both) is developmentally regulated. For example, T-lymphoma cell lines arrested at early stages of differentiation have low levels of *lck* transcripts; *lck* expression increases progressively to high levels in cells at developmental stages corresponding to those of mature thymocytes before decreasing to intermediate levels in peripheral blood T lymphocytes (11). In addition, the immature T-cell leukemia cell line, P30/OKUBO, can be induced to express high levels of *lck* transcripts by treatment with tetradecanoylphorbol acetate (TPA) (11). Furthermore, the levels of *lck* transcripts can be transiently down regulated in peripheral blood T lymphocytes induced to proliferate by treatment with TPA and phytohemagglutinin (PHA) (16).

Two distinct *lck* transcripts (denoted type I and II mRNAs), containing different 5′ untranslated region (5′ UTR) sequences, have been identified in the mouse (2, 8, 24). The sequence of the 3′ (or downstream) promoter, from which type I *lck* transcripts initiate, has been described (2, 8). However, neither the sequence of the 5′ (or upstream) promoter, from which type II *lck* transcripts initiate, nor the expression of the individual type I and II transcripts during T-cell development or activation has been determined. We report here the isolation and sequence of the two human *lck* promoter regions and the identification of the RNA start sites for the type I and II transcripts. Further, we show that (at least) the upstream *lck* promoter is differentially used during T-cell development and activation in vitro.

MATERIALS AND METHODS

Cells. Human T-cell leukemia (Jurkat, HSB-2, and P30/OKUBO) and colon carcinoma (COLO 201, COLO 205, COLO 320HSR, HT 29, and SW 948) cell lines were cultured in RPMI 1640 medium containing 10% fetal calf serum. Peripheral blood mononuclear cells were obtained from normal adult donors by Ficoll-Paque (Pharmacia, Inc., Piscataway, N.J.) centrifugation, and T lymphocytes were isolated by E rosetting with sheep erythrocytes. Human HeLa cells were cultured in alpha medium containing 10% fetal calf serum.

Chemicals. TPA (P-L Biochemicals, Inc., Milwaukee, Wis.), PHA (Wellcome Foundation, Ltd., Beckenham, England), and cyclosporin A (CsA) (Sandoz Canada, Inc., Dorval, Quebec, Canada) were used at final concentrations of 32 nM, 160 μg/ml, and 1 μg/ml, respectively, in RPMI 1640 medium containing 10% fetal calf serum.

Genomic library and library screening. A 2-kilobase (kb) *Eco*RI fragment containing the entire *lck* coding sequence and a 0.3-kb *Eco*RI-*Bgl*II fragment containing the 5′ UTR from the human *lck* cDNA clone YT16 (10) were used as probes for screening the genomic library of Maniatis et al. (14). Six clones were isolated; one hybridized to a synthetic oligonucleotide probe (30-mer) that spans nucleotides 36 to 7 upstream of the starting ATG codon in YT16 (10).

DNA sequencing. Relevant DNA fragments were isolated

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from the bacterial λ phage clones, digested with appropriate restriction enzymes, and subcloned into M13mp8 and M13mp9 for sequence analysis by using the Sanger dideoxy-chain termination technique (19).

Northern (RNA) blot analysis. Total cellular RNA was extracted from cells by homogenization in the presence of guanidine hydrochloride. Glyoxalated RNA (10 μg) was electrophoresed through a 1% agarose gel in 10 mM sodium phosphate buffer (pH 7.0), transferred to Gene Screen Plus (Dupont, NEN Research Products, Boston, Mass.), and then hybridized with the full-length human *lck* (YT16) and interleukin-2 (IL-2) nick-translated probes as described previously (11). The quality and quantity of RNA isolated from the various cell sources were verified by probing the Northern blots with a hamster α-tubulin probe.

S1 nuclease mapping. For the downstream *lck* promoter, a 321-base-pair (bp) *Fok*I-*Cla*I fragment ^{32}P-labeled at the *Cla*I site and complementary to positions −60 to +261 (see Fig. 2A) was used as an S1 probe. For the upstream promoter, a 197-bp *Hin*dIII-*Xma*I fragment ^{32}P-labeled at the *Xma*I site and complementary to positions −100 to +97 (Fig. 2B) was used as an S1 probe. Hybridization of 20 to 30 μg of total cellular RNA to 10,000 cpm of probe was carried out as described previously (26), except that the hybridization temperature was 50°C. Samples were treated with 150 U of S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at 20°C for 2 h. ^{32}P-labeled, S1 nuclease-protected products were processed as described previously (13) and then fractionated through 6% polyacrylamide–8 M urea sequencing gels. Autoradiography of the dried gels was performed with XAR film (Eastman Kodak Co., Rochester, N.Y.) and an intensifying screen (Cronex Lightning-Plus; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) at −70°C.

Primer extension analysis. A 30-mer 5′-^{32}P-end-labeled (25,000 cpm per reaction) oligonucleotide complementary to codons 7 to 16 of the *lck* coding region (see Fig. 2A) was hybridized to 20 to 30 μg of total cellular RNA at 37°C for 14 to 16 h and analyzed by primer extension exactly as described previously (7). Electrophoresis and autoradiography were performed as described above for S1 nuclease mapping.

RESULTS

Restriction map of the human *lck* gene. To characterize the sequences involved in regulating the expression of the human *lck* gene, we first isolated its 5′-flanking regions by screening the Maniatis human genomic library (14), using the human *lck* cDNA (YT16 [10]) as the probe. λ phage clones containing *lck* sequences were isolated, and restriction maps of the inserts were determined by using the enzymes *Eco*RI, *Hin*dIII, and *Pst*I. Figure 1 shows the restriction maps of two clones, λ1T (18.3 kb) and λ5G (15.3 kb), which hybridized to the YT16 probe. This probe hybridized with an 8-kb *Eco*RI subfragment of λ1T (p16G1T2N), and the 9.3 (p5G1)- and 4.8-kb *Eco*RI fragments of λ5G. In addition, a synthetic oligonucleotide probe (30-mer) representing the 5′ UTR of YT16 (Fig. 2A) hybridized to λ1T, whereas the coding region of YT16 and a different 5′ UTR (see below) are contained within λ5G. A distance of more than 9 kb separates these two 5′ UTRs of the human *lck* gene (Fig. 1).

Sequences of the two human *lck* promoter regions. Previous studies indicate that two *lck* transcripts, with different 5′ UTRs, are expressed in murine T cells (2, 8, 24). We therefore isolated and sequenced restriction fragments spanning the two human *lck* 5′ UTRs (Fig. 2). Figure 2A shows the sequence of the 0.7-kb *Pst*I fragment and part of the 6-kb *Pst*I fragment of p5G1 (Fig. 1), which contains the first coding exon, the 5′ UTR, and the 5′-flanking sequences of the downstream *lck* promoter. We note here that our sequence differs from the previously reported sequence (8) at 11 positions, including 9 nucleotide insertions (positions −547, −535, −524, −495, −492, −440, −408, −159, and −41) and 2 nucleotide substitutions (positions −449 and −385). The two RNA start sites for the type I *lck* transcripts (see below) are indicated, and the most frequently used (Ia) site was designated position +1. The splice acceptor and donor (4, 20) sites are located at positions +204 and +315, respectively. Figure 2B shows the sequence of the 1-kb *Eco*RI-*Pst*I fragment of p16G1T2N (Fig. 1), which contains the 5′ UTR of YT16 and the 5′-flanking sequences of the upstream *lck* promoter. The five RNA start sites for the type II *lck* transcripts (see below) are indicated, and the most frequently used (IIc) site was designated position +1. The splice donor (4, 20) site is located at position +107.

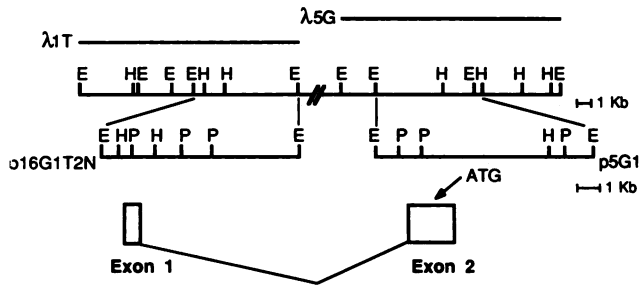

FIG. 1. Restriction enzyme map of the two promoter regions of the human *lck* gene. Clones λ1T and λ5G contain the 5′ or upstream promoter and 3′ or downstream promoter region, respectively. Subclones p16G1T2N and p5G1 are described in the text. The approximate positions of the exons and initiator ATG codon are indicated. Restriction enzymes are indicated as follows: E, *Eco*RI; P, *Pst*I; H, *Hin*dIII.

An examination of the 5′-flanking sequences of the two *lck* promoters did not reveal any readily identifiable consensus sequences corresponding to known eucaryotic *cis*-acting upstream promoter elements, such as TATA, CCAAT, or GC boxes (4, 7, 27). This search also failed to detect any strong homologies to typical enhancer motifs in either of the two *lck* promoters. However, Adler et al. (2) noticed that a sequence in the mouse type I *lck* promoter between positions −64 and −57 matches the immunoglobulin heavy-chain enhancer consensus sequence. This octanucleotide sequence (CCAGGTGG) is also present in the human type I *lck* promoter between positions −47 and −40 (Fig. 2A); this region has been shown to be highly conserved between human and mouse cells (8).

Transcription start sites of the human *lck* gene. To determine the RNA start sites of the *lck* gene, we used the methods of S1 nuclease and primer extension mapping. We analyzed total cellular RNA from a human T-cell leukemia cell line, Jurkat, which has been shown to constitutively express abundant amounts of *lck* transcripts (11). Total cellular RNA from human HeLa S-3 cervical carcinoma epithelial cells served as a negative control. We first used a 321-bp *Fok*I-*Cla*I fragment (Fig. 2A) labeled at the *Cla*I site as an S1 probe to detect type I *lck* transcripts initiated from the downstream promoter. Two S1 nuclease-protected fragments of approximately 261 and 241 bases in length and corresponding to start sites Ia and Ib (Fig. 2A), respectively,

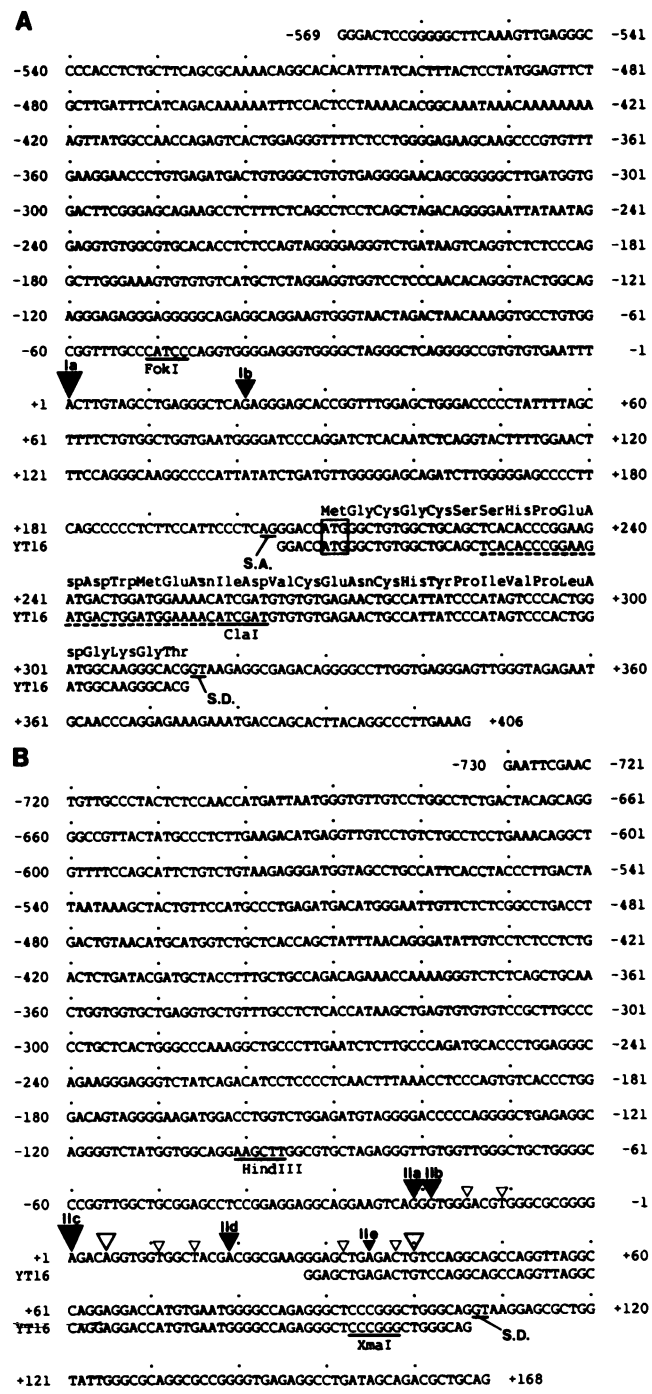

FIG. 2. Sequences of the human *lck* downstream (type I) promoter region (A) and upstream (type II) promoter region (B). (A) Sequence of 976 bp 3′ to the first *Pst*I restriction site within p5G1 (Fig. 1) spanning the downstream promoter. The most frequently used start site is numbered as position +1; this nucleotide is numbered as position −20 in reference 8. (B) Sequence of the 898-bp *Eco*RI-*Pst*I restriction fragment of p16G1T2N (Fig. 1) spanning the human *lck* upstream promoter. The most frequently used start site is numbered as position +1. Transcription start sites, determined by both S1 nuclease (Fig. 3) and primer extension (Fig. 4) mapping, are indicated (▼); the size of the inverted triangle indicates the relative start site usage in Jurkat cells. Start sites determined by S1 nuclease (but not primer extension) mapping are also indicated (▽). The splice acceptor (S.A.) and donor (S.D.) sites are indicated, and the nucleotide sequence of the human *lck* cDNA YT16 (10) is shown for comparison. The 30-mer oligonucleotide used for primer extension is indicated by a dashed underline, and the restriction enzyme sites used to prepare the S1 probes are indicated by a solid underline. The initiator ATG codon is boxed, and the amino acid sequence within the *lck* coding region is indicated.

were detected in Jurkat but not in HeLa cell RNA (Fig. 3A). The precise start site positions for the type I *lck* transcripts were determined by comparison of the S1 nuclease-protected fragments with the adjacent Sanger sequencing ladder (Fig. 3B). In addition, intense S1 nuclease-protected fragments of about 60 bases in length, which would be generated by hybridization of the S1 probe to the spliced type II *lck* transcripts arising from the upstream promoter, were detected in RNA from Jurkat but not from HeLa cells (Fig. 3A); this S1 probe is complementary to the human *lck* cDNA YT16 from positions +261 to +202 (Fig. 2A). The other bands detected in this S1 nuclease analysis were visible in both the Jurkat and HeLa cell lines (Fig. 3A).

We next used a 197-bp *Hin*dIII-*Xma*I fragment (Fig. 2B) labeled at the *Xma*I site as an S1 probe to detect type II *lck* transcripts initiated from the upstream promoter. This probe detected several S1 nuclease-protected fragments ranging from 118 to 58 bases in length, five of which correspond to start sites IIa to IIe (Fig. 2B), in RNA from Jurkat but not from HeLa cells (Fig. 3C). These S1 nuclease-protected fragments present in the RNA from Jurkat cells are likely to be T-cell-specific products, as bands of similar sizes were not detected in RNA from HeLa cells even upon longer exposure of the autoradiogram (Fig. 3C and data not shown).

The RNA start sites of the type I and II *lck* transcripts identified with S1 nuclease were confirmed by primer extension mapping. A 30-mer oligonucleotide was synthesized complementary to codons 7 to 16 (positions +228 to +257) of the *lck* coding region in YT16 (Fig. 2A); this 30-mer should anneal to both type I and II *lck* transcripts. We analyzed total cellular RNA from HeLa, Jurkat, and P30/OKUBO cells and from peripheral blood T lymphocytes. At least seven T-cell-specific primer extension products were observed with RNA from Jurkat but not from HeLa cells, ranging from 257 to 109 bases in length (Fig. 4). The precise start site positions of these *lck* transcripts were then determined by comparison of the primer extension products with the adjacent Sanger sequencing ladder (Fig. 4 and data not shown).

The primer extension products of 257 and 237 bases in length correspond to the type I *lck* transcripts detected as RNA initiated at the Ia and Ib start sites, respectively (Fig. 2A), and the primer extension products of 180 to 178, 159, 141, and 125 bases in length correspond to the type II *lck* transcripts detected as RNA initiated at the IIa/b, IIc, IId, and IIe start sites, respectively (Fig. 2B). The apparent discrepancy in sizes of bands generated by primer extension as compared with S1 nuclease mapping was due to differences in the positions of the 5′ ends of the primer and the S1 probes (Fig. 2). Thus, the primer extension products were 4 bases smaller or 62 bases larger than the corresponding S1 nuclease-protected fragments for the type I or II *lck* transcripts, respectively (Fig. 3 and 4). The relative intensities of the bands generated for each of the identifiable RNA start sites were roughly similar in the two assay systems (compare the Jurkat lanes in Fig. 4 with those in Fig. 3). The primer extension product of 109 bases in length, which was observed only in the T-cell lanes (Fig. 4), does not correspond to any band detected by S1 nuclease mapping. Similarly, S1

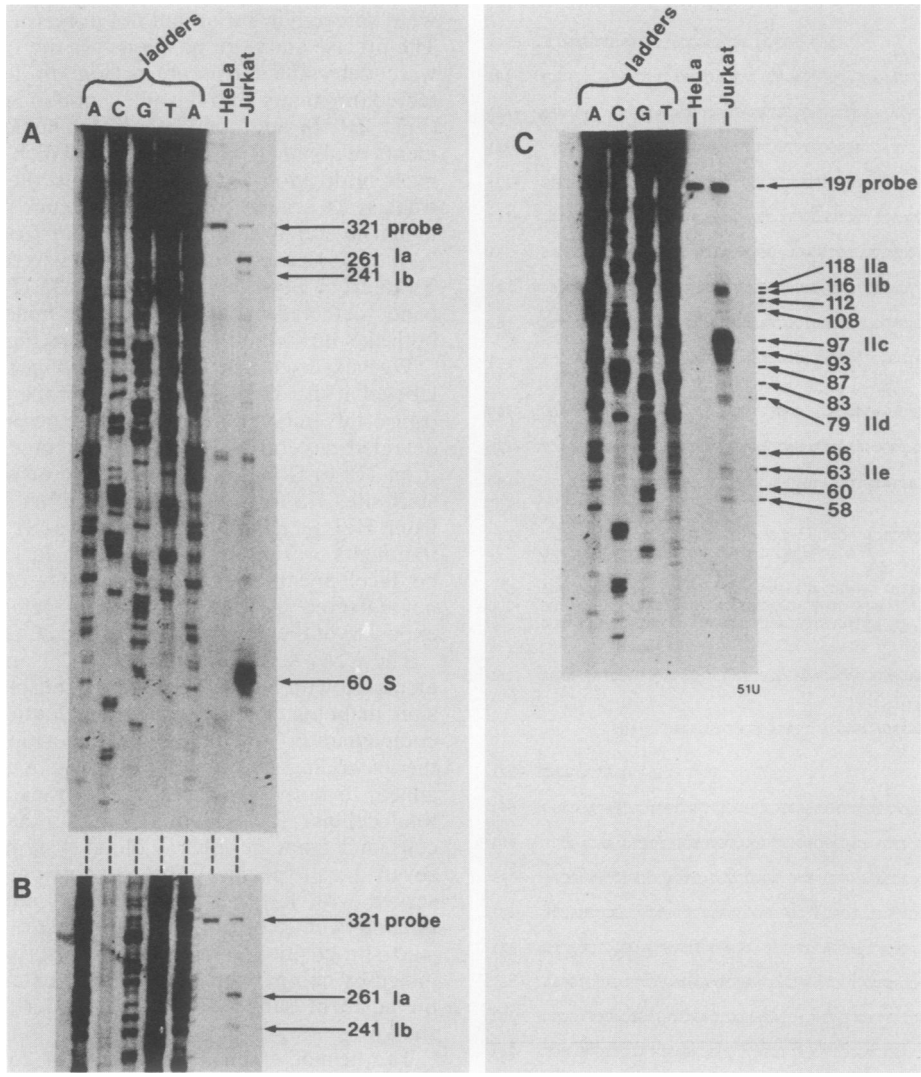

FIG. 3. S1 nuclease mapping of the human *lck* transcripts in Jurkat cells. Total cellular RNA from HeLa and Jurkat cells, as indicated at the top of the lanes, was hybridized to restriction fragments and 5′ ^{32}P end labeled on the RNA coding strand, and S1 nuclease digestion was performed as described in Materials and Methods. (A, B) S1 nuclease mapping with a probe spanning the downstream promoter region. The 321-bp *Fok*I-*Cla*I probe (Fig. 2A) and the S1 nuclease-protected fragments of approximately 261, 241, and 60 bases in length detected in Jurkat cells are indicated. Ia and Ib, Major and minor start sites, respectively, in the downstream promoter (their positions are shown in Fig. 2A); S, spliced transcripts that initiate in the upstream promoter (see text). To more precisely identify the positions of the start sites, the samples in panel A were electrophoresed for a longer time (B). (C) S1 nuclease mapping with a probe spanning the upstream promoter region. The 197-bp *Hin*dIII-*Xma*I probe (Fig. 2B) and the approximate sizes of the S1 nuclease-protected fragments detected in Jurkat cells are indicated. IIa to IIe refer to the start sites in the upstream promoter; their positions are shown in Fig. 2B. Ladders are Sanger dideoxy sequencing reactions (19) with the 30-mer oligonucleotide shown in Fig. 2 as the primer.

nuclease mapping identified several type I start sites (Fig. 3C and 2B [▽]) for which no corresponding primer extension products were detected (Fig. 4).

Modulation of *lck* transcripts during T-cell development. Previously we had shown that expression of *lck* is developmentally regulated during T-cell maturation. Northern blot analysis of total cellular RNA with the full-length human *lck* cDNA YT16 as the probe revealed that immature thymocytes and mature T cells express lower levels of *lck* than do more mature thymocytes (11). In addition, *lck* expression was shown to be inducible by phorbol esters, as exposure of the immature T-cell line, P30/OKUBO, to TPA for 24 h resulted in a marked increase in the levels of *lck* transcripts (11). It was of interest, therefore, to determine whether the modulation of *lck* expression during the development of T cells may involve the regulation of expression of *lck* transcripts that initiate from either a particular set of start sites or one of the two *lck* promoters.

The S1 nuclease-protected fragments of 261, 241, and 60 bases in length, which correspond to start sites Ia and Ib and spliced type II transcripts, respectively, were detected in RNA from HSB-2 cells, whereas only the spliced type II transcripts were present at detectable levels in untreated P30/OKUBO cells (Fig. 5). As expected from our previous work (11), primer extension and S1 nuclease mapping experiments revealed higher levels of *lck* transcripts of T-cell leukemia cells corresponding to mature thymocytes (Jurkat and HSB-2) than in immature thymocytes (P30/OKUBO) or peripheral blood T lymphocytes (Fig. 4 and 5, and data not shown). This higher level of *lck* expression in Jurkat and

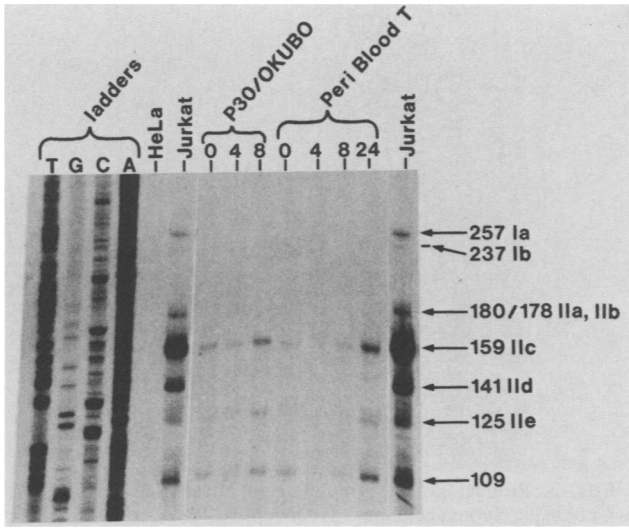

FIG. 4. Primer extension mapping of the human *lck* transcripts in Jurkat cells. Total cellular RNA from the sources indicated at the top of each lane were hybridized to the 5′-^{32}P-end-labeled 30-mer oligonucleotide primer complementary to the *lck* coding region (Fig. 2), and primer extension was performed as described in Materials and Methods. P30/OKUBO cells and peripheral blood T lymphocytes (Peri Blood T) were treated with TPA alone or TPA plus PHA, respectively, at concentrations described in Materials and Methods, for 0 to 24 h as indicated above the lanes. The approximate sizes of the primer extension products and the start sites that each of the bands represents (Fig. 2) are indicated. A Northern blot analysis of the RNA using the hamster α-tubulin probe (see Materials and Methods) indicated that the sample prepared from P30/OKUBO cells treated with TPA for 4 h was underrepresented (not shown). Sanger sequencing ladders were as described for Fig. 3.

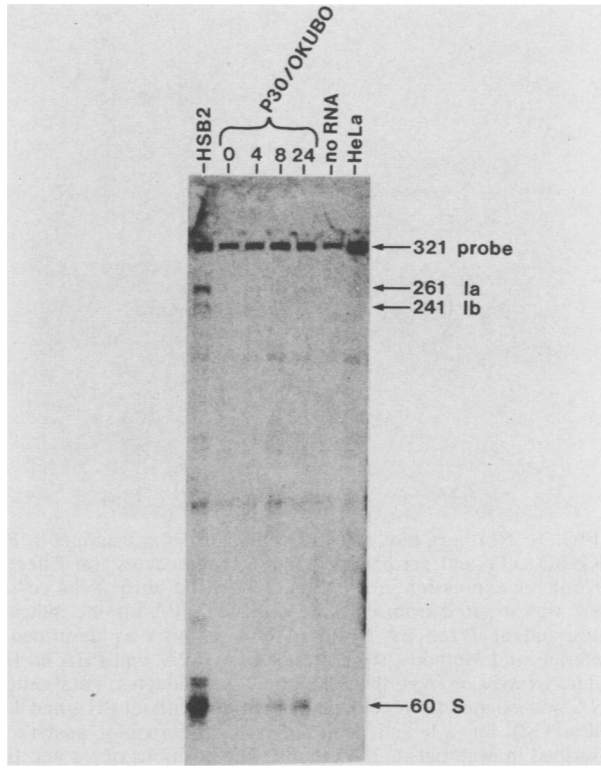

FIG. 5. S1 nuclease mapping of human *lck* transcripts in P30/OKUBO and HSB-2 cells. Total cellular RNA from the sources indicated at the top of each lane were analyzed by S1 nuclease mapping as described in the legend to Fig. 3. P30/OKUBO cells were treated with TPA as described in the legend to Fig. 4. The symbols used are as in Fig. 3. Upon longer exposure of this autoradiogram (not shown), the presence of an S1 nuclease-protected fragment of 261 bases in length was more clearly visible, and the additional S1 nuclease-protected fragments present in RNA from HSB-2 cells were observed in RNA from HeLa cells.

HSB-2 cells was due to an increase in steady-state levels of both type I and II transcripts, suggesting an increased activity of the two *lck* promoters. We also observed that type II transcripts are found in consistently greater abundance than type I transcripts, suggesting that the upstream promoter is more active than the downstream promoter. It should be noted that although the type I and II *lck* mRNAs were dissimilar only in their 5′ UTRs, it is possible that the steady-state levels of the type I and II transcripts do not accurately reflect the transcription rates from the downstream and upstream promoters, respectively.

Confirming our previous results (11), Fig. 6A shows that TPA induced the expression of *lck* at the steady-state RNA level and that this increase was detected within 4 h after addition of TPA. S1 nuclease and primer extension mapping experiments were then used to estimate the frequency of utilization of specific start sites in the two *lck* promoters in TPA-treated P30/OKUBO cells. A steady increase in the intensities of the S1 nuclease-protected fragment of 60 bases in length (Fig. 5) and the primer extension products of 159, 141, and 125 bases in length (Fig. 4) was observed upon exposure of the P30/OKUBO cells to TPA. A barely detectable S1 nuclease-protected fragment of 261 bases in length was also observed with RNA from TPA-treated (but not from untreated) P30/OKUBO cells (Fig. 5). However, bands corresponding to type I (Ia and Ib) or minor type II (IIa/IIb) transcripts were not detected by the primer extension analysis (Fig. 4). Nevertheless, these results suggest that the induction of *lck* expression in P30/OKUBO cells by TPA is mediated primarily by increases in the steady-state levels of type II transcripts.

Modulation of *lck* transcripts during T-cell activation. Recently, it has been reported that the level of *lck* transcripts in peripheral blood T lymphocytes declines transiently between 4 and 8 h after addition of PHA and TPA, and this modulation of *lck* expression closely correlates, in an inverse relationship, with the induction of lymphokine production (16). To determine the relative *lck* promoter usage in human peripheral blood T lymphocytes activated by PHA and TPA, we attempted to quantitate the *lck* transcripts initiating from both the upstream and downstream promoter start sites by primer extension. Figure 4 shows that the levels of the primer extension products corresponding to *lck* transcripts initiating from start sites IIc, IId, and IIe coordinately declined 4 to 8 h after addition of PHA plus TPA and increased by 24 h. Type I and IIa/IIb *lck* transcripts were not detected by this assay.

As CsA, an effective immunosuppressive agent, is known to inhibit the induction of interleukin-2 (IL-2) transcripts by PHA and TPA (6, 12), we examined the effect of CsA on the PHA-plus-TPA-mediated down regulation of *lck* transcripts. As expected from the work of Marth et al. (16), treatment of peripheral blood T lymphocytes for 4 h with PHA and TPA resulted in a reduction of *lck* transcripts that was coincident with the induction of IL-2 transcripts; this induction of IL-2 was indeed suppressed by cotreatment with CsA (Fig. 6B). Interestingly, CsA did not affect the reduction of *lck* tran-

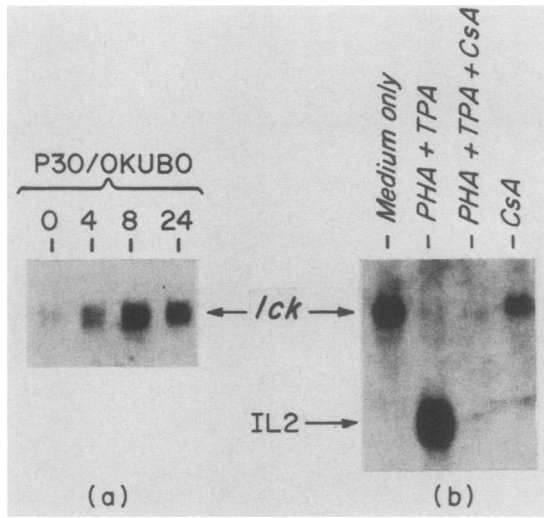

FIG. 6. Northern blot analysis of human *lck* transcripts in P30/OKUBO cells and peripheral blood T lymphocytes. (a) Effect of TPA on *lck* expression in P30/OKUBO cells in vitro. Total cellular RNA was isolated from cells treated with TPA for the indicated times and analyzed by Northern blot analysis as described in Materials and Methods. (b) Effect of CsA, TPA, and PHA on IL-2 and *lck* expression in peripheral blood T lymphocytes. Total cellular RNA was isolated from cells treated with or without PHA and TPA and/or CsA for 4 h and analyzed by Northern blot analysis as described in Materials and Methods. The positions of *lck* and IL-2 transcripts are indicated.

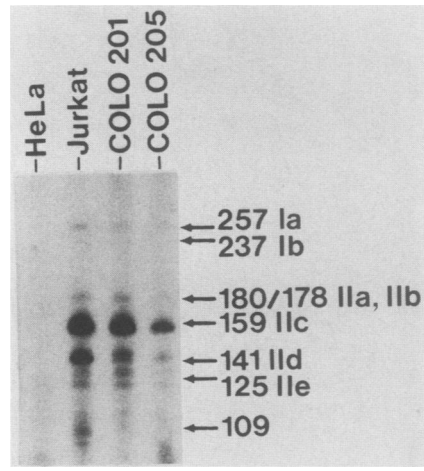

FIG. 7. Primer extension mapping of human *lck* transcripts in two colon carcinoma cell lines. Total cellular RNA from the sources indicated at the top of each lane were analyzed by primer extension mapping as described in the legend to Fig. 4. Symbols are as in Fig. 4.

scripts mediated by PHA and TPA (Fig. 6B). The lower steady-state levels of *lck* transcripts in peripheral blood T lymphocytes activated by PHA and TPA were reflected by a general decrease in all of the type II *lck* transcripts as determined by primer extension (data not shown).

Expression of *lck* transcripts in human colon carcinoma cell lines. In addition to its expression in T cells, the human *lck* gene is also expressed at high levels in a subset of colon carcinoma cells and at moderate levels in a variety of other nonlymphoid tumor cells (22). We performed primer extension mapping experiments with total cellular RNA from several human colon carcinoma cell lines. In cells that express *lck* at high levels, such as COLO 201 and COLO 205 (22), we detected type I and II *lck* transcripts in amounts similar to those observed in Jurkat T-cell leukemia cells (Fig. 7). We detected low amounts of type II transcripts in SW 948 cells, and we were unable to detect any *lck* transcripts in HT 29 cells (data not shown); both of these cell lines express *lck* at very low levels (22). We also detected *lck*-specific primer extension products in COLO 320HSR cells; however, the start sites used were different from those used in T cells (data not shown).

DISCUSSION

We have determined the structure of the human *lck* gene upstream and downstream promoter regions and mapped their transcription start sites. By S1 nuclease and primer extension mapping we have shown that, as in the mouse (2, 8, 24), two classes of *lck* transcripts are expressed in human T cells. In fact, at least two RNA start sites from the downstream promoter and five start sites from the upstream promoter can be detected. Transcripts initiating from the downstream and upstream promoters have been denoted type I and II *lck* transcripts, respectively (2, 24). The human type I *lck* transcripts initiated 210 and 189 bp upstream of the ATG translation initiation codon. The murine *lck* cDNA NT18 is derived from a transcript of this type (18). The type II *lck* transcripts initiated from a promoter located at least 9 kb from the downstream promoter in both human (Fig. 1) and mouse (2) cells and created an alternative 5′ UTR through splicing to 5 nucleotides 5′ to the initiation ATG codon. The human *lck* cDNA YT16 (10) and the murine *lck* cDNA pA2 (2) are derived from transcripts of this type.

The human type I *lck* transcript which initiated at start site Ib at position +21 (Fig. 2A) corresponds to the major start site identified in the murine *lck* downstream promoter (2, 8). Interestingly, the major type I start site used in human T cells (Ia) has been described as a possible minor start site in the murine *lck* downstream promoter (2). Of potential importance is the observation that a 4-bp gap was inserted into the mouse sequence at the human type I transcript Ia start site (8) to align the sequences of the human and mouse *lck* downstream promoters. It will be of interest to determine whether this difference in start site utilization is involved in regulating *lck* expression from the downstream promoter.

We analyzed the sequences of the human *lck* upstream and downstream promoters by using the Sequence Analysis Software Package from the Genetics Computer Group at the University of Wisconsin. From this analysis, it appeared that the 5′-flanking regions of the two human *lck* promoters are unrelated. Furthermore, we were unable to identify any consensus sequences for known eucaryotic *cis*-acting elements, such as the TATA box, which is responsible for the positioning of the mRNA start sites or level of transcription from a promoter (or both) (4), or for other regulatory elements usually located in the proximal 5′-flanking region of RNA polymerase II promoters (27). It is possible that the lack of consensus TATA box sequences in both the upstream and downstream promoter regions is responsible for the multiple type I and II RNA start sites.

In the Moloney murine leukemia virus-induced murine thymoma cell line, LSTRA, Moloney murine leukemia virus has been inserted 969 bp 5′ to the type I promoter start site (2, 8). A hybrid long terminal repeat-*lck* transcript is expressed in this cell line by splicing of RNA, initiated at the viral long terminal repeat start site, from the splice donor in

the viral leader to the *lck* splice acceptor 5 nucleotides 5′ to the initiator ATG codon. Thus, the hybrid long terminal repeat-type II *lck* transcripts use the same splice acceptor to generate functional *lck* mRNAs. This retroviral promoter insertion results in the overexpression of *lck* transcripts that lack the normal 5′ UTR; the hybrid long terminal repeat-*lck* transcript is efficiently translated in vivo (17). It is noteworthy that in the 5′ UTR of the normal murine *lck* downstream promoter, the presence of three AUG sequences upstream of the initiator AUG codon has been shown to reduce the efficiency of $p56^{lck}$ translation (17). Two of these AUG sequences, at +78 and +148, are conserved in the human type I *lck* transcripts. The former is in frame with the initiator AUG codon such that a peptide of 44 additional amino acids could be produced. The human type II *lck* transcripts also have two out-of-frame upstream AUG sequences, at +71 and +77, interrupted by a TGA termination codon. It will be of interest to determine whether these AUG sequences in the 5′ UTR of the human *lck* type I and II mRNAs play a role in regulating $p56^{lck}$ translation.

In the human T-cell lines tested (Jurkat, HSB-2, and P30/OKUBO) and in human peripheral blood T lymphocytes, type II *lck* transcripts were found in greater abundance than type I *lck* transcripts, suggesting that the upstream *lck* promoter is more active than the downstream *lck* promoter. In agreement, it has been shown by Northern blot analysis with type I- and II-specific probes that type II *lck* transcripts are found in greater relative abundance in human thymus than in Jurkat cells (8). In fact, we were unable to detect, by primer extension, type I *lck* transcripts in either P30/OKUBO cells or peripheral blood T lymphocytes. It will be necessary to use more-sensitive assays, such as RNase protection, to detect *lck* transcripts that initiate from either the type I or minor type II RNA start sites. In contrast with our findings, it has been reported recently that the relative usage of two *lck* promoters varies considerably in certain murine T-cell lines (24). For example, transcripts that initiate from the type II *lck* promoter are barely detected in BW5147 thymoma cells, whereas they appear to predominate in the helper T-cell lines E5 and L2. It will be of obvious interest to analyze RNA from other human T-cell lines, as well as from subsets of T thymocytes and peripheral blood T lymphocytes, to determine whether the relative usage of the two *lck* promoters correlates with the expression of specific T-cell surface markers or with different functional subsets of T cells (see below) or both.

The expression of *lck* is maintained at a higher level in thymocytes and at a relatively lower level in peripheral blood T lymphocytes and immature T cells (11). Here we demonstrate that the higher steady-state levels of *lck* transcripts in Jurkat and HSB-2 cells (leukemic T cells corresponding to an intermediate stage of thymic differentiation) are due to increases in both the type I and II *lck* transcripts. Interestingly, exposure of the T-cell line P30/OKUBO, which is arrested at an early stage of thymic differentiation (germ line α, β, and γ chain T-cell receptor genes), to TPA resulted in an increased utilization of the upstream and (to a lesser extent) downstream promoter start sites. Moreover, the type II *lck* transcripts derived from the upstream promoter responded to T-cell activation by PHA and TPA in peripheral blood T lymphocytes, although in this case the type I *lck* transcripts could not be detected. Nevertheless, it is possible that the efficiency of transcription from the human *lck* upstream and downstream promoters is regulated independently during certain physiological states.

Previous studies have shown that *lck* transcripts are transiently down regulated (16) and $p56^{lck}$ is posttranslationally modified (23) in T lymphocytes activated with PHA and TPA. We observed that the reduction of *lck* transcripts upon activation of T lymphocytes by PHA and TPA was not affected by the treatment of cells with CsA and PHA plus TPA, whereas as expected (6, 12), induction of IL-2 transcripts was clearly blocked (Fig. 6). This result is consistent with the idea that the down regulation of *lck* transcripts is necessary but not sufficient for the induction of IL-2 and other lymphokines during T-cell activation.

Two human colon carcinoma cell lines, COLO 201 and COLO 205, expressed type I and II *lck* transcripts at high levels, whereas other human colon carcinoma cell lines, COLO 320HSR, SW 948, and HT 29, expressed either low or undetectable levels of *lck* transcripts. Moreover, the relative start site usage in COLO 201 and COLO 205 was similar to that seen in Jurkat or HSB-2 cells. Veillette et al. (22) have shown that *lck* transcripts are more abundant in colon carcinoma cell lines derived from lymph node metastases or ascites, such as COLO 201 and COLO 205, compared with those cell lines established from primary tumors, such as COLO 320HSR, SW 948, and HT 29. Finally, it has been reported recently that the T-lymphocyte CD4 and CD8 proteins, which are expressed on the surface of functionally distinct populations of T cells, both bind to $p56^{lck}$ (21). Thus, the expression of *lck* in colon carcinoma cells is intriguing in view of the observation that CD4, which is felt to represent the human immunodeficiency virus receptor on T helper cells, is also expressed in some colon carcinoma cells (1). Therefore, expression of *lck* is likely to be central to the development of thymocytes or the activation of T cells or both and may be important in the progression of some nonlymphoid tumors as well. The elucidation of the molecular mechanisms regulating *lck* transcription and translation will be beneficial towards a further understanding of the cellular role(s) of $p56^{lck}$.

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LITERATURE CITED

1. **Adachi, A., S. Koenig, H. E. Gendelman, D. Daugherty, S. Gattoni-Celli, A. S. Fauci, and M. A. Martin.** 1987. Productive, persistent infection of human colorectal cell lines with human immunodeficiency virus. J. Virol. **61**:209–213.
2. **Adler, H. T., P. J. Reynolds, C. M. Kelley, and B. M. Sefton.** 1988. Transcriptional activation of *lck* by retrovirus promoter insertion between two lymphoid-specific promoters. J. Virol. **62**:4113–4122.
3. **Bishop, J. M.** 1985. Viral oncogenes. Cell **42**:23–38.
4. **Breathnach, R., and P. Chambon.** 1981. Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**:349–383.
5. **Cooper, J. A., K. L. Gould, C. A. Cartwright, and T. Hunter.** 1986. Tyr 527 is phosphorylated in $pp60^{c\text{-}src}$: implications for regulation. Science **231**:1431–1434.
6. **Elliott, J. F., Y. Lin, S. B. Mizel, R. C. Bleackley, D. G. Harnish, and V. Paetkau.** 1984. Induction of interleukin 2 messenger RNA inhibited by cyclosporin A. Science **226**:1439–1441.

7. **Frederickson, R. M., M. R. Micheau, A. Iwamoto, and N. G. Miyamoto.** 1989. 5′ flanking and first intron sequences of the human β-actin gene required for efficient promoter activity. Nucleic Acids Res. **17**:253–270.
8. **Garvin, A. M., S. Pawar, J. D. Marth, and R. M. Perlmutter.** 1988. Structure of the murine *lck* gene and its rearrangement in a murine lymphoma cell line. Mol. Cell. Biol. **8**:3058–3064.
9. **Hunter, T., and J. A. Cooper.** 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. **54**:897–930.
10. **Koga, Y., N. Caccia, B. Toyonaga, R. Spolski, Y. Yanagi, Y. Yoshikai, and T. W. Mak.** 1986. A human T cell-specific cDNA clone (YT16) encodes a protein with extensive homology to a family of protein tyrosine kinases. Eur. J. Immunol. **16**:1643–1646.
11. **Koga, Y., N. Kimura, J. Minowada, and T. W. Mak.** 1988. Expression of the human T-cell-specific tyrosine kinase YT16 (*lck*) message in leukemic T cell lines. Cancer Res. **48**:856–859.
12. **Kronke, M., W. J. Leonard, J. M. Depper, S. K. Arya, F. Wong-Staal, R. C. Gallo, T. A. Waldmann, and W. C. Greene.** 1984. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. Proc. Natl. Acad. Sci. USA **81**:5214–5218.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Maniatis, T., R. C. Hardison, E. Lacy, J. Laver, C. O'Connell, D. Quon, G. K. Sim, and A. Efstratiadis.** 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell **15**:687–702.
15. **Marth, J. D., J. A. Cooper, C. S. King, S. F. Ziegler, D. A. Tinker, R. W. Overell, E. G. Krebs, and R. M. Perlmutter.** 1988. Neoplastic transformation induced by an activated lymphocyte-specific tyrosine kinase (pp56^{lck}). Mol. Cell. Biol. **8**:540–550.
16. **Marth, J. D., D. B. Lewis, C. B. Wilson, M. E. Gearn, E. G. Krebs, and R. M. Perlmutter.** 1987. Regulation of pp56^{lck} during T-cell activation: functional implications for the *scr*-like protein tyrosine kinases. EMBO J. **6**:2727–2734.
17. **Marth, J. D., R. W. Overell, K. E. Meier, E. G. Krebs, and R. M. Perlmutter.** 1988. Translational activation of the *lck* proto-oncogene. Nature (London) **332**:171–173.
18. **Marth, J. D., R. Peet, E. G. Krebs, and R. M. Perlmutter.** 1985. A lymphocyte-specific protein-tyrosine kinase gene is rearranged and overexpressed in the murine T cell lymphoma LSTRA. Cell **43**:393–404.
19. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
20. **Sharp, P. A.** 1981. Speculations of RNA splicing. Cell **23**:643–646.
21. **Veillette, A., M. A. Bookman, E. A. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56^{lck}. Cell **55**:301–308.
22. **Veillette, A., F. M. Foss, E. A. Sausville, J. B. Bolen, and N. Rosen.** 1987. Expression of the *lck* tyrosine kinase gene in human tumor cell lines. Oncogene Res. **1**:357–374.
23. **Veillette, A., I. D. Horak, E. M. Horak, M. A. Bookman, and J. B. Bolen.** 1988. Alterations of the lymphocyte-specific protein tyrosine kinase (p56^{lck}) during T-cell activation. Mol. Cell. Biol. **8**:4353–4361.
24. **Voronova, A. F., H. T. Adler, and B. M. Sefton.** 1987. Two *lck* transcripts containing different 5′ untranslated regions are present in T cells. Mol. Cell. Biol. **7**:4407–4413.
25. **Voronova, A. F., and B. M. Sefton.** 1986. Expression of a new tyrosine protein kinase is stimulated by retrovirus promoter insertion. Nature (London) **319**:682–685.
26. **Wasylyk, B., C. Kedinger, J. Corden, O. Brison, and P. Chambon.** 1980. Specific *in vitro* initiation of transcription on conalbumin and ovalbumin genes and comparison with adenovirus-2 early and late genes. Nature (London) **285**:367–373.
27. **Wingender, E.** 1986. Compilation of transcription regulating proteins. Nucleic Acids Res. **16**:1879–1902.